Preliminary Studies on Establishment of Genetic Transformation System in Loblolly Pine

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Abstract A transformation system was established for loblolly pine (*Pinus taeda* L.) mature zygotic embryos using *Agrobacterium tumefaciens*. The gene coding for the β-glucuronidase (GUS) gene was introduced into loblolly pine tissues and its transient expression was detected with histochemical staining. The influences of different genotypes, *Agrobacterium* concentrations, and cocultivation time on GUS expression and kanamycin resistant callus and shoot regeneration were investigated. The results showed that the highest GUS expression frequency (16.3%) and shoot regeneration frequency (7.8%) were obtained from genotype 9-1003 with *Agrobacterium* concentration decreased 9 times and cocultivation time of 56 hours, respectively. GUS expression was obtained in all genotypes tested. The successful expression of the GUS gene in different genotypes suggested that it will be a useful transformation system for loblolly pine.

Key words: *Pinus taeda* L., Genetic transformation, *Agrobacterium tumefaciens*

Introduction

Conifers are economically important forest tree worldwide, being used for soil conservation, environment protection, wood production, and as a variety of building and industry material. For conifer genetic improvement plan, classical breeding programs and gene transfer techniques are available. However, classical breeding programs are rather slow and tedious because of the long generation times and the inability to introduce specific genes when crossing parental lines. Genetic transformation and recombined DNA technology can provide an alternative, direct and rapid method for the introduction of specific genes in conifers[1, 2, 3]. Stable genetic transformation has been developed for a lot of forest tree species and used to transfer agronomically interesting genes conferring traits such as virus, insect or herbicide resistance[4], but the transformation of conifers has only been reported recently and is restricted to a few species[5].

Inoculation with *Agrobacterium tumefaciens* produced tumor development in numerous coniferous species[3, 5]. Transgenic plant regeneration has only been obtained recently in hybrid larch[6]. On the other hand, *Agrobacteririum rhizogenes* was also used successfully to regenerate transgenic larch plantlets expressing genes for herbicide and insect resistance[4]. Transgenic plant regeneration in other conifers was not obtained, mainly due to the lack of a suitable regeneration procedure[5]. Because of difficulties using *Agrobacterium*, attempts to transform conifers have been reported using direct gene transfer methods such as particle bombardment and electroporation. Transient expression was observed in electroporated protoplasts of white spruce and Douglas fir[7], as well as *Pinus taeda*, *Picea abies*, and *Picea mariana* embryogenic tissues after particle bombardment[4]. Stable transformation was reported in *Picea abies* after bombardment of somatic embryos (without plantlet regeneration)[8]. Recently, Ellis et al[5] reported successful transformation and transgenic plant regeneration of *Picea glauca*, and Charest et al[9] also reported similar results for *Picea mariana*. In the present investigation, we reported the stable transformation of loblolly pine mature zygotic embryos using *Agrobacterium tumefaciens* and the factors affecting transformation frequency of gene. This study may lead to

the development of *Agrobacterium tumefaciens*-mediated transformation procedure and transgenic plant regeneration of loblolly pine.

Materials and Method

Plant Materials

Mature cones of eight different genotypes of loblolly pine were collected from Shaoyang Seed Orchard (Hb, Ma and Mc), Hunan Province, Yingde Seed Orchard (82-2, 83-11, and 84-40), Guangdong Province, and South America (7-506, 9-1003) in October, 1994 to 1996, respectively, and stored at 4°C icebox until use. Mature zygotic embryos were isolated from megagametophyte before surface-sterilized in 0.1% mercuric chloride for 8 min followed by three successive rinses in sterile water, and were cultured on DCR medium[10] containing 2 mg/L NAA and 0.5 mg/L BA for 3-5 days, then used to transformation experiment.

Agrobacterium strain and cocultivation

Cocultivation experiment were conducted using the *Agrobacterium tumefaciens* LBA 4404 harboring the plasmid pBI121 carrying neomycin phosphotransferase (NPII) gene, which confers kanamycin resistance, and β -glucuronidase (GUS) gene. The GUS DNA sequence was under the control of the cauliflower mosaic virus 35S promoter and the terminator from nopaline synthase (nos) provides poly (A) signal. Transformation of mature zygotic embryos was obtained after cocultivation of zygotic embryos with *Agrobacterium tumefaciens*. Bacteria were grown for one day at 28 °C on liquid YEP medium supplemented with the appropriate antibiotics, then the bacterium concentration was decreased 5-13 times and used in the infection of embryos about 5-8 min. Cocultivation was conducted at 25 °C for 24-72 h in the darkness on DCR medium with NAA and BA.

Selection and regeneration

Cocultivated mature zygotic embryos were washed 3-5 times in sterile distilled water for eliminating the *Agrobacterium tumefacines*, followed by a cefotaxime (250 mg/L) wash for 20 min, following which they were extensively washed in sterile distilled water. Zygotic embryos were placed on sterile paper to remove excess liquid and transferred onto differentiation medium supplemented with 500 mg/L cefotaxime and 15 mg/L kanamycin. After three weeks, kanamycin-resistant embryos were isolated and kept under selective pressure, during all of the following steps until callus and adventitious shoot regeneration. Differentiation medium consisted of DCR medium containing 2 mg/L BA, 0.5 mg/LIBA, 500 mg/L casein hydrolysate, and 500 mg/L glutamine. All media supplemented with 3% sucrose and 0.7% agar. The pH was adjusted to 5.8 prior to autoclaving for 30 min at 115 °C. Differentiation culture of mature zygotic embryos was conducted at 25 °C under a 16 h photoperiod. Light density is 1500 Lx.

Detection of GUS gene expression and scanning electron microscopy

Histochemical analysis of GUS gene expression was performed on mature zygotic embryos as described by Jefferson[11] and Cheng et al[12]. Kanamycin resistant embryos were counted and GUS gene expression embryos were recorded in the third week after cocultivation. Callus formation on mature zygotic embryos was monitored 12 weeks after inoculation. Shoot regeneration on zygotic embryos was recorded 18 weeks after inoculation. Mature zygotic embryos, callus and adventitious shoot were prepared for scanning electron microscopy according to Fowke et al[13] with some modification. Embryos and tissues were fixed overnight in 4%(v/v) glutaraldehyde and 100 mM phosphate buffer, pH 7.0, washed one time in 100 mM phosphate buffer (pH 7.0) for 30 min, followed by dehydration in successive ethyl alcohol solution of 85, 90, 95 and 100%, each repeated twice for 30 min. Specimens were dried in a critical-point-drier with CO_2 for 2 h, mounted on Cu stubs and gold-coated. The samples were examined and photographed in HITACHI scanning electron microscope. All experiments were repeated thrice, and each treatment consisted of 30-50 mature zygotic embryos.

Results and Discussion

Regeneration of transgenic calli and shoots

3-5 days after culturing on DCR medium with NAA and BA, mature zygotic embryos were cocultivated with *Agrobacterium tumefaciens* LBA 4404 harboring plasmid pBI121 carrying neomycin phosphotransferase (NPT II)gene and β-glucuronidase (GUS) gene. After cocultivation, explants were put on DCR differentiation medium containing 15 mg/L kanamycin as a selective agent. The toxic level of this antibiotics to non-transformed mature zygotic embryos had been determined in preliminary experiments. In six out of eight tested genotypes, transient GUS expression was de-

tected 10 days after cocultivation. Three weeks after cocultivation, GUS expression was detected in all tested genotypes. The scanning electron microscopy showed that callus was initiated on cotyledons of mature zygotic embryos in third week after cocultivation (Fig. 1). Kanamycin resistant roots were formed on the radicle in 4th week after cocultivation (Fig. 2). Six weeks after cocultivation, calli were formed on the hypocotyl in all tested genotypes (Fig. 3). Primary adventitious buds derived from callus were observed in the 15th week after cocultivation (Fig. 4). 0.5-0.9 mm shoots from callus ere obtained in the 18th week (Fig. 5). 0.3-0.5 cm kanamycin resistant shoots were observed in the 21st week after cocultivation (Fig. 6).

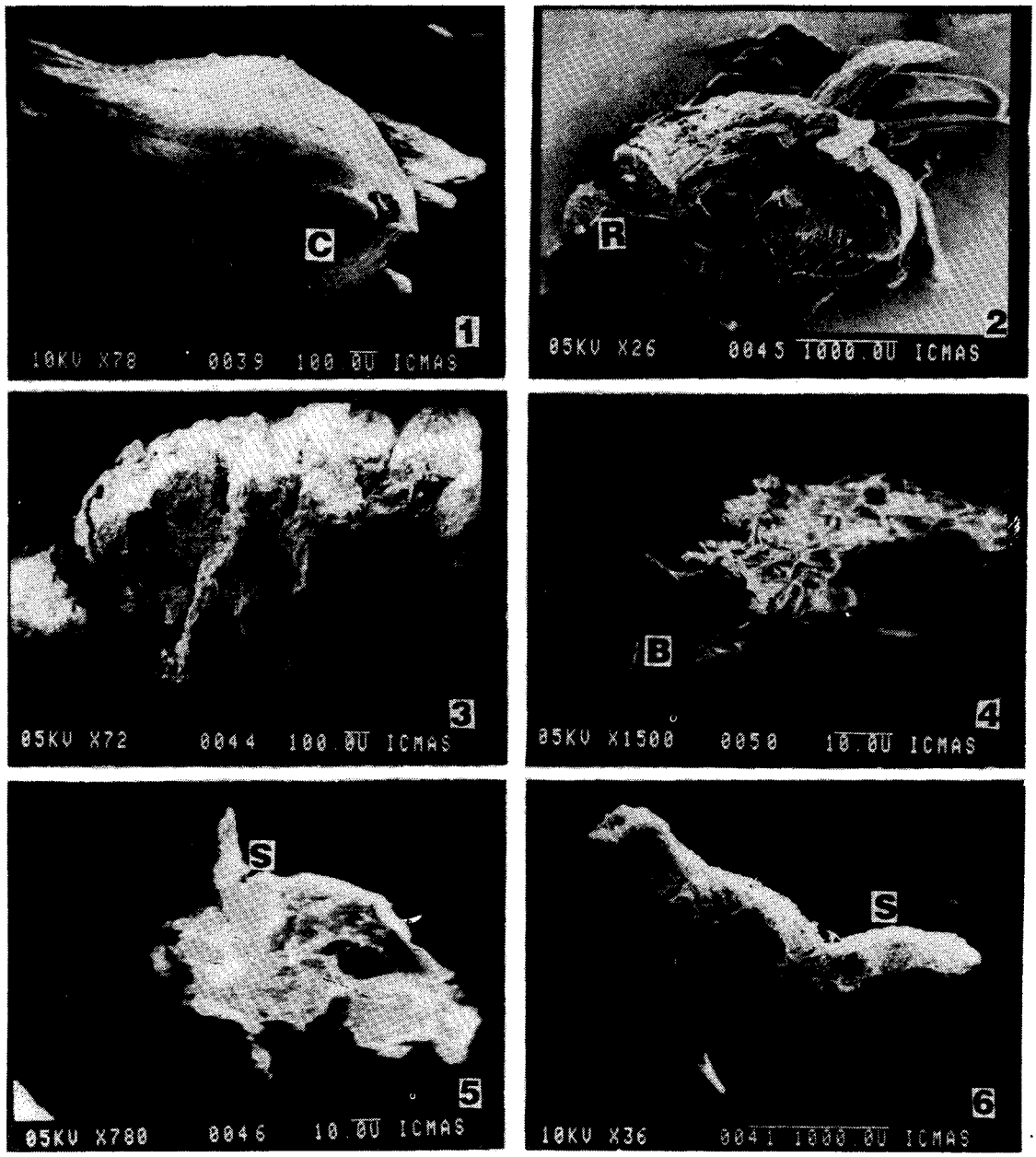

Fig. 1. Initiation of callus (C) from cotyledons of mature zygotic embryos of loblolly pine. Fig. 2. Regeneration of kanamycin resistant root (R). Fig. 3. Kanamycin resistant callus derived from hypocotyl. Fig. 4. Formation of primary adventitious buds (B). Fig. 5. Kanamycin resistant shoot (S) induced from callus expressing GUS activity. Fig. 6. Transgenic shoot (S) derived from genotype 9-1003.

Influence of genotypes and bacterium concentration on transformation

For establishing a general transformation system suitable for loblolly pine, eight genotypes were tested. The results showed that genotype 7-56 and 9-1003 had the higher frequency of embryos of kanamycin resistance GUS expression, callus formation, and shoot regeneration in the tested genotypes (Table 1). Differences of introduced gene expression among genotypes were also confirmed on white spruce[14] and white pine[15] The results of bacterium concentration experiments showed that transformation frequency was higher when the concentration decreased 9-11 times, and genotype 7-56 and 9-1003 had the highest kanamycin resistant shoot regeneration frequency (data not show).

Table 1 Influence of different genotypes on transformation in loblolly pine

Genotypes	Percentage of mature zygotic embryos(%)			
	Kanamycin resistance	GUS expression	Callus formation	Shoot regeneration
11b	12.6 ± 0.2	11.2 ± 0.9	9.1 ± 0.7	1.3 ± 0.1
Ma	13.2 ± 0.7	11.7 ± 0.8	9.3 ± 0.8	1.7 ± 0.2
Mc	14.1 ± 0.4	12.1 ± 1.2	9.7 ± 0.8	2.8 ± 0.1
7-56	19.7 ± 0.3	17.8 ± 1.2	13.6 ± 1.1	5.6 ± 0.2
9-1003	20.8 ± 1.5	18.3 ± 1.7	14.2 ± 1.2	7.1 ± 0.3
82-2	14.5 ± 1.1	11.4 ± 0.9	9.8 ± 0.8	3.1 ± 0.2
83-11	13.9 ± 0.9	10.9 ± 0.8	9.4 ± 0.8	2.4 ± 0.1
84-40	13.3 ± 1.2	10.7 ± 1.0	8.6 ± 1.0	1.9 ± 0.1

Note Values represent the means ± S.D

Influence of cocultivation time on transformation

To determine whether the cocultivation time of *Agrobacterium tumefaciens* with mature zygotic embryos influenced NPT II gene expression, GUS gene expression, callus formation, and shoot regeneration, different cocultivation times were conducted using mature zygotic embryos of genotype 9-1003 as explants of transformation. The results showed that the frequency of embryos kanamycin resistance, GUS gene expression, callus formation, and shoot regeneration were the highest, 19.14%, 16.3%, 14.7%, and 7.8%, respectively, when the cocultivation time was 56h (Table 2). High frequency of GUS gene expression and shoot regeneration were also observed when cocultivation time was 48h. In other treatment, the frequency of GUS expression was low. Duchesne et al[16] also reported the similar results in the transformation of embryogenic callus of black spruce.

Table 2 Influence of cocultivation time on transformation of loblolly pine

Genotypes	Percentage of mature zygotic embryos(%)			
time (h)	Kanamycin resistance	GUS expression	Callus formation	Shoot regeneration
24	10.1 ± 0.9	9.7 ± 0.7	7.2 ± 0.8	1.7 ± 0.1
32	11.3 ± 1.0	9.8 ± 0.9	8.1 ± 0.7	2.3 ± 0.1
40	13.7 ± 1.2	10.5 ± 1.0	10.3 ± 0.9	2.5 ± 0.1
48	18.2 ± 2.1	15.6 ± 1.3	13.6 ± 1.2	6.4 ± 0.2
56	19.4 ± 2.2	16.3 ± 1.3	14.7 ± 1.3	7.8 ± 0.3
64	14.5 ± 1.0	9.4 ± 1.0	9.1 ± 0.9	3.2 ± 0.1
72	12.6 ± 0.9	9.1 ± 0.9	8.4 ± 0.8	2.9 ± 0.1

Note Values represent the means ± S.D

At the beginning of this work, a very good regeneration system through organogenesis was available for different genotypes of loblolly pine and seems to be an important precondition for successful transformation. The transformation system described in this article appears to be easily transferable to all genotype explants tested, since eight genotypes tested gave rise to kanamycin resistant calli and shoots. It now remains to be seen whether this transformation system can be successfully adapted to other coniferous species where a good regeneration system already established.

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